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STUDIES ON THE BIOCHEMISTRY OF SULPHUR

VI. THE CYSTINE CONTENT OF CONPHASEOLIN AND PHASEOLIN, THE ALPHA AND BETA GLOBULINS OF THE NAVY BEAN (*Phaseolus vulgaris*)

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STUDIES ON THE BIOCHEMISTRY OF SULPHUR

VI. THE CYSTINE CONTENT OF CONPHASEOLIN AND PHASEOLIN, THE ALPHA AND BETA GLOBULINS OF THE NAVY BEAN (PHASEOLUS VULGARIS)

In previous work on phaseolin, the beta globulin of the navy bean, Sullivan, in Paper V of this series, found this globulin contained little, if any, cystine. Since the sample of phaseolin used was some 7 or 8 years old, left from the work of Waterman, Johns, and Jones (1923), it was decided to repeat the work with freshly made samples of phaseolin and also of conphaseolin, the alpha globulin of the navy bean. The results are embodied in the present paper and at the same time notice is called to the cystine content of the alpha and beta globulins of other beans of the *Phaseolus* genus, such as the mung bean, the adsuki bean, and the lima bean.

Osborne (1894) isolated from the navy bean two proteins—phaseolin, a globulin soluble in 2 per cent aqueous sodium chloride, and another protein phaselin, much more soluble in dilute salt solution than phaseolin. Osborne did not assign phaselin to any class. Of the bean meal, phaseolin, according to Osborne, comprised approximately 21.5 per cent, phaselin 2 per cent. Later, Waterman, Johns, and Jones (1923) found a small amount of a second globulin, which they called conphaseolin, the alpha globulin present in the bean meal and to the extent of about 0.4 per cent.

Though Osborne obtained phaseolin in crystalline form by dialytic fractionations, the alpha and beta globulins obtained by Waterman, Johns, and Jones were amorphous.

The new globulin, conphaseolin, is early precipitated out of the 2 per cent salt solution by the addition of ammonium sulphate, and is therefore called the alpha globulin. Phaseolin, the beta globulin, requires much more ammonium sulphate for precipitation. The beta globulin comprises the main portion of the protein of the navy bean. The alpha globulin differs from phaseolin and phaselin most conspicuously by its very much higher sulphur content.

As indicated by Waterman, Johns, and Jones, it is somewhat difficult to separate these two globulins of the navy bean so that the conphaseolin contains no phaseolin and the phaseolin, in turn, contains no conphaseolin.

In their work these investigators found that the phaseolin obtained by the ammonium sulphate method had a lower sulphur content than preparations made by Osborne by dialytic fractionations and considered that their sample was more nearly free from conphaseolin.

It would seem from a comparative study of their phaseolin, designated old phaseolin in this article, that they effected a rather thorough separation. This old preparation of phaseolin was employed in the cystine studies outlined in paper 5 of this series. In the present paper comparisons are made of old and new preparations of phaseolin and of conphaseolins as well, and striking differences between the alpha and beta globulins are brought out. Of the original conphaseolin of Waterman, Johns, and Jones very little was left, so this compound is given a separate treatment later in this paper.

The fresh samples of the alpha globulin and the beta globulin were tested for cystine by means of the Sullivan procedure found satisfactory for casein as recently described (Sullivan 1929). Five-gram samples of conphaseolin, old phaseolin, new phaseolin, and leached casein were hydrolyzed 6 hours with 20 per cent hydrochloric acid, decolorized, neutralized, diluted to 100 c. c. and tested for cystine following procedures given in paper No. 5 of this series. In the hydrolysis, the phaseolin and conphaseolin gave considerable humin, the casein gave slight humin. In the colorimetric work, the old phaseolin gave a pale yellow color, indicative of little, if any, cystine in the hydrolysate; the new sample of phaseolin showed slight red in an orange solution, suggestive of the presence of a little cystine; while the conphaseolin, on the contrary, gave a strong test for cystine—decidedly red. Casein gave a fair red.

The indication of cystine in the new phaseolin was so slight that the only way it could be estimated satisfactorily was by using the phaseolin hydrolysate to dilute a standard cystine solution and noting the increase in color over the cystine standard similarly diluted with 0.1 N hydrochloric acid.

This procedure tends to give somewhat higher results than the actual, since there is a tendency to read the added yellow put into the cystine solution by the phaseolin as an intensity factor when matched against the standard cystine. Using appropriate standards for conphaseolin and casein and estimating the cystine in the phaseolin by difference, the cystine content of the four samples, old phaseolin, new phaseolin, conphaseolin, and leached casein, was found to be as given in Table 1.

TABLE 1.—*The percentage cystine content of conphaseolin, phaseolin, and casein*

Substance	Cystine found
Old phaseolin	0.00
New phaseolin	.11
Conphaseolin	1.18
Casein	.28

The cystine content of conphaseolin and phaseolin (old) was determined by Waterman, Johns, and Jones (1923) by means of the Van Slyke method. Conphaseolin gave 1.18 per cent cystine, and phaseolin gave 1.16 per cent cystine. Our findings with conphaseolin agree with the findings of the above-mentioned investigators employing Van Slyke's method. With phaseolin, however, there is absolute disagreement, as shown in Table 1 and subsequent tables.

In the work, the results of which are given in Table 1, the decolorizing agent was purified norite. Over 2 grams of norite were required to decolorize the hydrolysate of 5 grams of phaseolin and conphaseolin. Accordingly, to keep the results comparative, the same amount of norite was used with the casein hydrolysate and with 25 mg. of cystine put through the same procedure as the foodstuffs. Compared with a cystine solution containing 200 parts per million of cystine in 0.1 N hydrochloric acid, the recovery of cystine put through the hydrolyzing, treatment with norite, neutralizing, etc., was 72 per cent.[1]

This correction was applied to the findings with phaseolin, conphaseolin, and casein, and the figures given in Table 1 are corrected figures, but not further corrected for moisture and ash. The work was then repeated with the use of 0.4 gram of "carboraffin," a decolorizing agent which we have found much more efficient than norite and with less tendency to hold cystine.[2] The results, using carboraffin, and employing the Sullivan method, the Okuda (1925) iodometric method, and the Folin-Looney (1922) method for estimating cystine are given in Table 2. Column (1) under each method gives the percentage of cystine as found in the hydrolysate. Column (2) gives the percentage after correction for loss of cystine put through the whole procedure, heating, treatment with carboraffin, neutralizing, etc.

TABLE 2.—*The percentage cystine content of phaseolin and conphaseolin*

Substance	Sullivan method		Okuda method		Folin-Looney method	
	(1)	(2)	(1)	(2)	(1)	(2)
Old phaseolin	0.00	0.00	0.09	0.10	0.15	0.17
New phaseolin	[a].13	.14	.25	.28	.41	.46
Conphaseolin	1.09	1.21	1.20	1.33	1.78	1.99
Casein	.28	.31	.31	.34	.29	.32

[a] An approximation, probably too high. Direct comparison was attempted against a 100 p. p. million standard with readings far apart.

As shown in Table 2, the three methods are close together for casein. In many determinations on the cystine content of casein, the Sullivan and the Okuda methods give closely parallel results,

[1] Normally with exactly 2 grams of norite the recovery of cystine put through the procedure is 80 to 85 per cent.

The recovery of 25 milligrams of cystine put through the whole procedure with the use of carboraffin is 90 to 95 per cent.

but the Folin-Looney method has often given higher values. These points are illustrated in Table 4. In the case of the phaseolin and conphaseolin, as shown in Table 2, there is more or less divergence, especially in case of the Folin-Looney method. The divergence may be due to the degree of specificity of the methods. The Sullivan method is more specific for cystine than the Okuda and the Folin-Looney methods. Thus, as shown in papers 2 and 3 of this series, the Folin-Looney method not only reacts to various disulphides, such as cystine amine and the disulphide of thiotyrosine, but even with non-sulphur compounds, such as furfural and pyruvic acid. The Okuda method reacts positively also to various disulphides. In the present case of the phaseolins as given in Table 2 the Okuda method is closer to the Sullivan method.

The Okuda and the Folin-Looney methods give positive reactions with peptide sulphur, such as glutathione, so there was a slight possibility that the somewhat lower results with the Sullivan method were due to incomplete hydrolysis. Accordingly, a 36-hour hydrolysis was run with carboraffin as decolorizing agent. The results are given in Table 3, with correction for loss of cystine put through the same procedure in Column (2).

TABLE 3.—*The percentage cystine content of phaseolin and conphaseolin—36-hour hydrolysis*

Substance	Sullivan		Okuda	
	(1)	(2)	(1)	(2)
Old phaseolin	0.00	0.00	0.09	0.10
New phaseolin	.10	.11	.15	.16
Conphaseolin	1.01	1.12	1.15	1.28
Casein	.28	.31	.28	.31

The 36-hour hydrolysis gave, if anything, slightly lower results in the Sullivan and Okuda procedures and brought the results by these methods nearer together. No determinations were made by the Folin-Looney method. There seems to be no advantage in the longer hydrolysis over the 6-hour period.

HYDROLYSIS WITH INHIBITION OF HUMIN FORMATION

In the hydrolysis of both phaseolin and conphaseolin considerable humin was formed, acid soluble and acid insoluble, especially the latter. As previously described in papers 4 and 5, and as will be given in greater detail in a later paper by Sullivan and Hess, it is possible to hydrolyze purified proteins in a reducing atmosphere with little, if any, humin formation, certainly with no acid insoluble humin and with but a slight degree of coloration. To test out the possibility that cystine was lost in the formation of humin or that the insoluble

humin might have absorbed the cystine, the phaseolins were hydrolyzed under conditions which gave little if any humin. The procedure employed is briefly described.

To 1 gram each of phaseolin old, phaseolin new, conphaseolin, and leached casein were added 5 c. c. of 20 per cent hydrochloric acid and 1.5 c. c. of 20 per cent titanous chloride ($TiCl_3$). The mixtures in small acetylation flasks were heated for 1, 3, and 6 hours, respectively, at 125° C. Then the hydrolysates were poured into small beakers, the flasks washed with approximately 5 c. c. of water, and the solutions carefully neutralized with 5 N sodium hydroxide until the titanium was precipitated. The solutions reacted yellow to thymol blue, blue to brom phenol blue. They were filtered by suction through a small Buchner funnel containing one layer of hardened filter paper. All the solutions gave a direct nitroprusside reaction, indicative of the presence of cysteine. Air was blown through the four solutions for one hour, at the end of which time the direct nitroprusside reaction was negative. The reaction was then adjusted to pH 3.5 with concentrated hydrochloric acid and the volume made to 30 c. c. with 0.1 N hydrochloric acid. The standard was cystine dissolved in 0.1 N hydrochloric acid, so the figures are uncorrected. The results of analyses for cystine by the Sullivan, the Okuda, and the Folin-Looney methods are given in Table 4.

TABLE 4.—*The percentage of cystine in phaseolin, conphaseolin, and casein hydrolyzed without humin formation*

Substance	Sullivan method			Okuda method			Folin-Looney method		
	1 hour	3 hours	6 hours	1 hour	3 hours	6 hours	1 hour	3 hours	6 hours
Old phaseolin	0.00	0.00	0.00	0.07	0.09	0.09	0.29	0.26	0.50
New phaseolin	[1].10	[1].10	[1].14	.14	.18	.14	.55	.50	.79
Conphaseolin	.66	1.10	1.06	.98	1.11	1.15	1.40	1.50	1.90
Casein	.28	.28	.29	.30	.29	.30	.46	.53	.83

[1] An attempt was made to get a direct reading, but the standard and the new phaseolin were so far apart, with more yellow in the phaseolin, that the reading is only an approximation, probably much too high.

As shown previously (Sullivan 1929) in the second paper of the series, the Sullivan reaction has a greater degree of specificity than has any other method so far developed for cysteine or cystine. At all times with this method the indications have been that the sample of phaseolin made by Waterman, Johns, and Jones contains little, if any, cystine.

The recently made sample of phaseolin was found by the Sullivan method to contain a small amount of cystine, 0.10–0.13 per cent. Since casein with a cystine content of 0.28–0.30 per cent tends to be a protein deficient in cystine, as shown by a number of feeding experiments, the new phaseolin fed on the same level as that on which casein is usually fed, 18 per cent of the diet, would have to be rein-

forced by cystine to support the life of a white rat. In this respect both samples of phaseolin—old phaseolin and new phaseolin—agree; they are proteins deficient in cystine. The question at issue, however, is why the new sample shows more cystine than the old sample. The difference between the two samples may have two explanations. First, there may have been more or less oxidation of the old sample in the 8 years since it was isolated. Since the protein was stored in dry condition in a closed bottle, this possibility seems somewhat remote. The second explanation—undoubtedly the true cause of the difference between the two samples—is that the old sample was free from conphaseolin which is rich in cystine, while the new sample contained conphaseolin as an impurity. The question as to whether the sulphur in these proteins is oxidized by long keeping seemed worthy of further study. Accordingly, a small sample of conphaseolin, isolated in 1922 and especially purified by Jones for anaphylactic studies (Wells, Lewis, and Jones, 1927) was withdrawn from the "Protein Museum" of the Protein Investigation Laboratory for comparison with the sample made in April 1929.

The procedure in this comparative analysis was as follows: 500 milligrams each of the new conphaseolin (1929) and the old conphaseolin (1922) were heated with 5 c. c. of 20 per cent hydrochloric acid for six hours in an oil bath at 125° C. The hydrolysates were diluted with 5 c. c. of distilled water, decolorized by bringing to gentle boiling with 25 milligrams of carboraffin and filtered. The carboraffin was extracted by warming to gentle boiling with 5 c. c. of normal hydrochloric acid and filtered. The combined filtrates were brought to pH 3.5 by addition of 5 N sodium hydroxide, dropwise, with stirring, and the solution was made to 30 c. c. The colorimetric results by the Sullivan, the Okuda, the Folin-Looney and the Folin-Marenzi methods are given in Table 5.

TABLE 5.—*The percentage cystine content of old and new samples of conphaseolin*

Substance	Sullivan method	Okuda method	Folin-Looney method	Folin-Marenzi method
Conphaseolin 1922	1.20	1.32	1.19	.....
Conphaseolin 1929	1.18	1.31	1.18	1.19

In Table 5, the Sullivan and the Folin-Looney methods agree. Though such agreement between these methods is not the rule, it is here an experimental fact.

All the methods agree in showing that the cystine content of the sample of conphaseolin, isolated in 1922, is the same as that isolated in 1929.

In this case, at least, there is no oxidation of the cystine by long keeping. The difference between the old phaseolin and the new phaseolin must be due to the fact that the old phaseolin is a purer sample. This explanation has a high degree of probability, since considerable time and attention must be given to the fractionation of these practically amorphous compounds to obtain a sample of phaseolin free from conphaseolin and vice versa. The difference in the reaction of the hydrolysates of phaseolin and conphaseolin in the Sullivan procedure is so striking it would seem to indicate a method of differentiating between these globulins and to afford a test of the purity of the beta fractions in addition to the anaphylactic reaction suggested by Waterman, Johns, and Jones (1923).

THE BETA GLOBULINS OF OTHER BEANS OF THE GENUS PHASEOLUS

With the conclusion that the alpha globulin, or conphaseolin, of the navy bean is rich in cystine while the beta globulin, phaseolin, either contains none or so little that it is lost in the procedure of hydrolysis, the question arises as to the application of this finding to alpha and beta globulins of other beans of this genus. Similar alpha and beta globulins have been isolated from the mung bean by Johns and Waterman (1920); from the adsuki bean by Jones and others (1922), and from the lima bean by Jones and others (1922). The lima bean was found nutritionally lacking in cystine by Finks and Johns (1921), and the bean meal and the total globulins of the adsuki bean were found nutritionally deficient in cystine by Johns and Finks (1921). In a detailed study of the cystine content of purified proteins obtained from D. Breese Jones, Sullivan, and Hess, in work shortly to be published, determined the cystine content of 1-gram samples of the beta globulin of the mung bean, the adsuki bean, and the lima bean. The results of these analyses, employing the customary way of hydrolizing, decolorizing by carboraffin, etc., are given in Table 6.

TABLE 6.—*The percentage cystine content of the beta globulin of the mung bean, the adsuki bean, and the lima bean*

Substance	Sullivan method	Okuda method	Folin-Looney method
Beta globulin mung bean	0.04	0.10	0.25
Beta globulin adsuki bean	.01	.03	.28
Beta globulin lima bean	.13	.18	.32

The results with the Sullivan method and the Okuda iodometric method are in fair agreement and show that these beta globulins are deficient in cystine, in keeping with the results of feeding experiments. The Folin-Looney method, however, shows the presence of considerable cystine—a finding not in keeping with the findings of feeding experiments.

ALPHA GLOBULINS OF THE MUNG, ADSUKI, AND LIMA BEAN

Very little of these globulins, as isolated by Johns and Waterman, Jones and others, was obtainable. In order to get some idea of the cystine content of these proteins, 500 milligrams of each were hydrolyzed with 5 c. c. of 20 per cent hydrochloric acid for six hours, decolorized, neutralized in the customary way and brought to a volume of 25 c. c. The cystine was then determined by the Sullivan method, the Okuda iodometric method, and by the recent Folin-Marenzi (1929) improvement on the Folin-Looney method. All the samples gave considerable black insoluble humin on hydrolysis. Since there was not enough of these alpha globulins on hand to repeat the testing with and without humin formation or longer time of hydrolysis, the results as given in Table 7 are regarded as approximations only.

TABLE 7.—*The percentage cystine content of the alpha globulins of the mung bean, the adsuki bean, and the lima bean*

Substance	Sullivan method	Okuda method	Folin-Marenzi method
Alpha globulin mung bean	0.50	0.70	0.53
Alpha globulin adsuki bean	.97	1.24	1.22
Alpha globulin lima bean	1.35	1.90	2.08

As may be seen from Tables 6 and 7, all these methods agree in showing that the alpha globulins are relatively rich in cystine while the beta globulins contain much less of this essential amino acid.

Since the beta globulins form the major portion of the protein of these beans, the beans as a whole would tend to be deficient in cystine. Accordingly, they should be used in diets in association with foodstuffs containing an abundance of cystine, such as meat, fish, and eggs.

SUMMARY

Employing the highly specific Sullivan method, it was found that conphaseolin, the alpha globulin of the navy bean, contains over 1 per cent cystine, while phaseolin, the beta globulin, if free from the alpha globulin, contains little, if any, cystine—a chemical finding in keeping with the results of feeding experiments. The beta globulins of the mung bean, the adsuki bean, and, to a degree, the lima bean are also deficient in cystine. The alpha globulins on the contrary are relatively rich in cystine. Since the protein of these beans is mainly the beta globulin, the beans as a whole would tend to be deficient in cystine. Accordingly, in general diets, these beans should be used with foodstuffs rich in cystine, such as meat, fish, and eggs.

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